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14. ABSTRACT

A major barrier to understanding breast cancer is the lack of comprehensive and systematic large scale studies that provide functional information about the entire genome. These insights can be obtained through RNAi (RNA interference) genetic studies RNAi is a cellular process that regulates gene expression in a sequence specific manner. We have developed a library of plasmids expressing shRNAs that engage the endogenous RNAi pathway and produce mature siRNAs that efficiently target any gene of interest. We have generated more than 200,000 constructs that allow us to perform loss of function studies of almost every gene in the human genome. Furthermore, we have developed a microarray-based analytical platform that facilitates the study of thousands of genes concurrently in pools. We conducted a screen to detect resistance to anoikis (cell death trigged by loss of attachment to the extracellular matrix, ECM) in the MCF10A breast epithelial cell line. Our screen of 1,500 shRNAs resulted in identifying the well known tumor suppressor, Pten, as an attenuator of anoikis among other candidate genes. In addition, we have validated an in vitro anoikis assay as an approach to identify putative tumor suppressors involved in breast epithelial cell transformation.

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Cancer, RNAi, gene silencing

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Introduction:

RNA interference (RNAi) is a conserved biological process in response to double-stranded RNA (dsRNA)[1]. DsRNAs are processed into short interfering RNAs (siRNAs), about 22 nucleotides in length, by the RNase enzyme Dicer. The siRNAs are then incorporated into a silencing complex called RISC (RNA-induced silencing complex), which identifies and silences complementary messenger RNAs. The most well characterized source of endogenous triggers for the RNAi machinery are the microRNA genes[2,3]. Numerous studies have demonstrated that, in animals, miRNAs are transcribed to generate long primary polyadenylylated RNAs (pri-miRNAs)[4,5]. Through mechanisms not yet fully understood, the pri-microRNA is recognized and cleaved at a specific site by the nuclear Microprocessor complex[6-10] to produce a ~70-90 nucleotide microRNA precursor (pre-miRNA) which is exported to the cytoplasm [11,12]. Only then is the pre-miRNA recognized by Dicer and cleaved to produce a mature microRNA. This probably involves recognition of the 2 nucleotide 3' overhang created by Drosha to focus Dicer cleavage at a single site ~22 nucleotides from the end of the hairpin[13].

This process can be programmed experimentally in order to repress the expression of any chosen gene. We have constructed shRNA libraries (shRNA-mir) that uses our advanced understanding of miRNA biogenesis. ShRNA-mirs are modeled after endogenous miRNAs, specifically contained in the backbone of the primary miR-30 microRNA[14]. We have produced and sequence-verified more than 200,000 shRNAs covering almost all of the predicted genes in the mouse and human genomes[15].

Our shRNA library can function in both individual cell based assays and pooled screens. We have linked a unique 60-nuclotide DNA barcode to each shRNA vector to allow us to follow the fate of shRNAs in populations of virally transduced cells. If, for example, a particular shRNA provided resistance to a growth inhibitor stimulus, then the representation of its associated barcode should be increased after treatment. If a given shRNA sensitized a population to a specific stress, then the relative abundance of its barcode should diminish after the stress. This is measured by hybridizing genomic PCR products containing the barcodes to custom microarrays that contain the complement of these sequences. One can assess cellular response to different treatments by comparing barcode representations of cell populations expressing known shRNA. The development of this highly efficient RNAi library together with the ability to screen

pools of genes, provide us with the unique opportunity to investigate the entire genome in loss of function studies to find relevant genes to any biological process.

Cancer is responsible for almost a quarter of the total annual number of deaths in the US. Among them, breast cancer presents the highest incidence in women[16]. To understand the tumorigenic process, it is imperative to identify and characterize the genes that provide tumor cells with the capabilities requisite for their initiation and progression. A major barrier to increasing our understanding of breast cancer is the lack of comprehensive and systematic large scale functional studies. We address this by conducting a genome-wide in vitro RNAi screen to find genes that promote hallmarks of transformation like resistance to apoptosis or uncontrolled proliferation [17].

The best in vitro system to uncover genes involved in breast cancer would be one in which normal human mammary epithelial cells (HMEC) could be transformed by a single experimentally-induced genetic lesion. Several cell culture models of human cell transformation have been described in which primary human cells are first immortalized and then transformed by combinations of dominantly acting cellular and viral oncogenes[18-20]. Immortalization is achieved by viral oncogenes that alter the genetic background of the primary cells mainly by blocking TP53 and RB. Then, transformation is promoted by an additional single genetic lesion. However, blocking of these major tumor suppressors introduces a great bias in the screen, as it may mask other potentially relevant genes that show epistatic interactions with them. Furthermore, viral protein may interfere with a large number of cellular components, making it difficult to define a limited number of genetic lesions that cooperate for transformation.

The MCF-10A cell line represents an alternative to these classic models of human cell transformation. MCF-10A is a spontaneously immortalized, but non-transformed, human mammary epithelial cell line[21]. These cells exhibit numerous features of normal breast epithelium, including lack of tumorigenicity in nude mice, lack of anchorage-independent growth, and dependence on growth factors and hormones for proliferation and survival[21]. Furthermore, these cells retain the ability to form arrested acini that recapitulate the structure found in in vivo mammary glandular epithelium. Genetically, MCF-10A has been extensively characterized. These cells have a deletion of the locus containing p16 and p14ARF[21,22], but more importantly, they maintain intact p53 and Rb loci and a stable karyotype (S. Muthuswamy

personal communication and our own unpublished results). I have collaborated with Jose Silva, a postdoc in our lab on developing RNAi screens. We looked for genes that enhance resistance to apoptosis is examined by inducing anoikis in MCF-10A. Anoikis is a natural mechanism used by monostratified epithelia for tissue morphogenesis and as a defense mechanism against abnormal proliferation that is generally lost in transformed cell. After detachment from the extracellular matrix (ECM), normal cells die by anoikis while tumor cells survive[23,24]. Thus, the study of anoikis, a naturally occurring phenomenon in vivo using the MCF-10A cell line will provide us with a list of candidates with potential involvement in breast carcinogenesis. The most promising resulting genes will be further studied and their mechanism of action characterized. The data originated by these studies will increase our knowledge about breast tumorigenesis and may have an important impact on breast cancer therapy.

Body:

In mammalian cells, proliferation is the result of a highly complex signaling network. External and internal signals converge in every individual cell in order to maintain the tissue homeostasis and, as a final goal, the organism viability. Every cell integrates these control signals as a process to sense its environment and then decide whether to proliferate, arrest or die[25]. This elaborate homeostasis control is universally disturbed in cancers, and it leads to unrestrained proliferation, resistance to programmed cell death or both[17].

Using our pooled shRNA strategy, I assisted Jose Silva, a postdoc in our lab to perform a screen using a library of 1,500 shRNAs to monitor the acquisition of these tumor features for human normal breast epithelial cells. Cell death was induced by loss of attachment to the ECM. This process known as anoikis is widely used for the morphogenetic development of epithelial tissues. Cells that loose the contact with the ECM die by apoptosis, leaving an empty space that is used to remodel the organ. Additionally, anoikis is also a defense mechanism that has to be circumvented by epithelial tumor cells in order to grow out of their natural environment[23,24]. To induce anoikis, mammary epithelial MCF-10A cells expressing the shRNA library were plated in conditions that do not allow them to form ECM. These cells are sensitive to loss of attachment and die after several days. However, the activity of specific shRNAs allows the cells to gain resistance and prolong survival (*Figure.1*). Before performing the screen, we reconstructed the

experiment to test whether we could enrich the survival population of our anoikis assay with a known clone of resistant cells. In order to obtain a clone resistant to anoikis, MCF-10A cells were transformed with an activated form of Ras (MCF-10A/Ras). MCF10A/Ras and MCF-10A wild type were mixed in a frequency of 1/1,500 and the anoikis screen was performed. After the completion of the anoikis screen the representation of the MCF-10A/Ras cells was analyzed by allele specific quantitative PCR (QPCR). This experiment revealed that MCF-10A/RAS cells were enriched around 50 fold in the population that survived anoikis (data not shown).

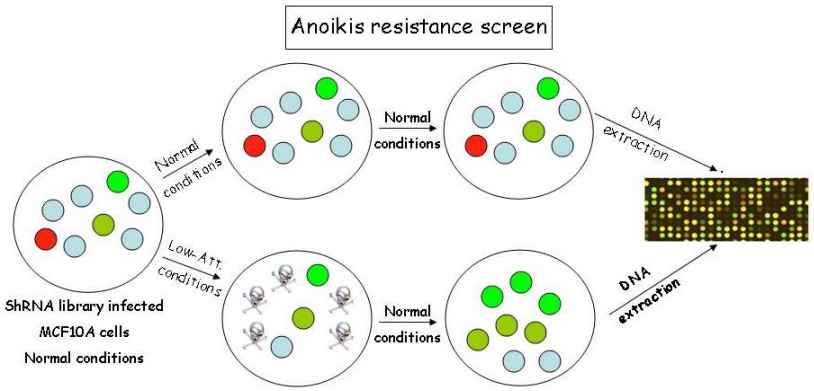

Figure 1. Anoikis screen protocol. *MCF-10A cells were infected with a library of 1,500 shRNAs. After puromycin selection, cell expressing hairpins were moved to ultra-low attachment plates for 5 days and grown back again in normal conditions for a week, or grown in normal conditions during the entire experiment. In low attachment conditions more than 95% of the cells died. The majority of the shRNAs of the library did not produce any changes in the apoptotic response (blue circles). A number of cells exhibited enhanced survival or increased sensitivity to cell death (green circles). The changes in the representation of each hairpin were monitored by comparing the DNAs of the anoikis stressed cells and the cells grown under normal conditions as described in the text.*

In addition, cells that had Pten knockdown were overrepresented in the population after anoikis stress (*Figure.2*). Interestingly, we also found that inhibition of Rb enhanced the cell death mediated by loss of attachment (*Figure.2*). It is important to mention that the screen was performed in triplicate, and that the st.dev. of the biological replicas was less than 30% for up to 80% of the hairpins.

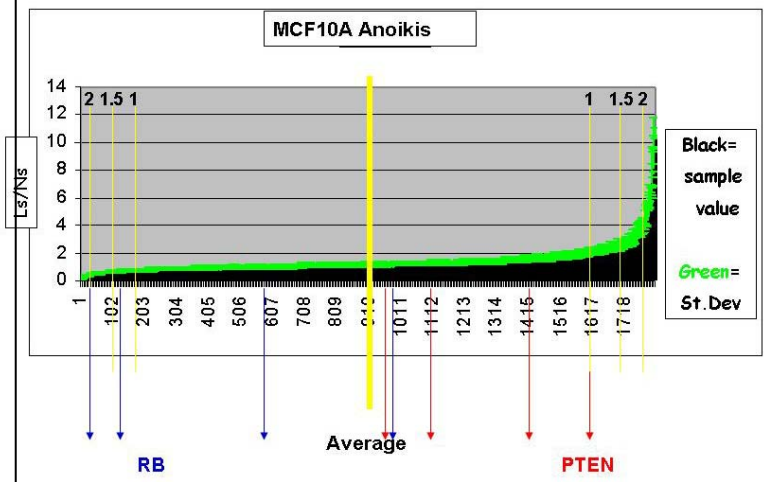

Figure 2. Anoikis screen results. *This graph shows the changes in the representation of 1,500 shRNAs after an anoikis assay of MCF-10A cells. The changes are quantified as the ratio*

between the representation after anoikis assay (Ls) and normal conditions of growth (Ns) . The bold yellow line shows the average of this ratio for the population of shRNAs and the thin lines represent the different possible cut offs (1, 1.5 and 2 st. dev). Green bars are the st.dev. for every individual barcode among the biological replicas. The arrows indicate the position of different hairpins targetting Rb (blue) and Pten (red) before and after puromycin selection.

Additionally, it is worth mentioning that barcode specific QPCR analysis confirmed the microarray results (data not shown). When Pten and Rb stably knocked-down cells were studied, we found that in MCF-10A cells, RNAi of Pten increased survival to anoikis 3 fold, and that RNAi of Rb reduced survival 2 fold (*Figure.3A and B*).

The tumour-suppressor phosphatase with tensin homology (PTEN) has been identified as one of the most important negative regulators of the cell-survival signaling pathway. When PTEN is not functional, augmented PI3K activity constitutively activates survival signals increasing the resistance to cellular stress. Numerous types of tumors have been found carrying alterations in PTEN[26]. Interestingly, Rb is another bona fide tumor suppressor that represses the expression of the transcription factor E2F. Derepressed E2F induces the transition of genes required for cell cycle progression[27]. Thus, it could be expected that suppression of Rb led to E2F downstream targets activation and induction of proliferation. However, E2F is also connected to apoptotic pathways by increasing the transcriptional level of caspases. Consequently, loss of Rb sensitizes

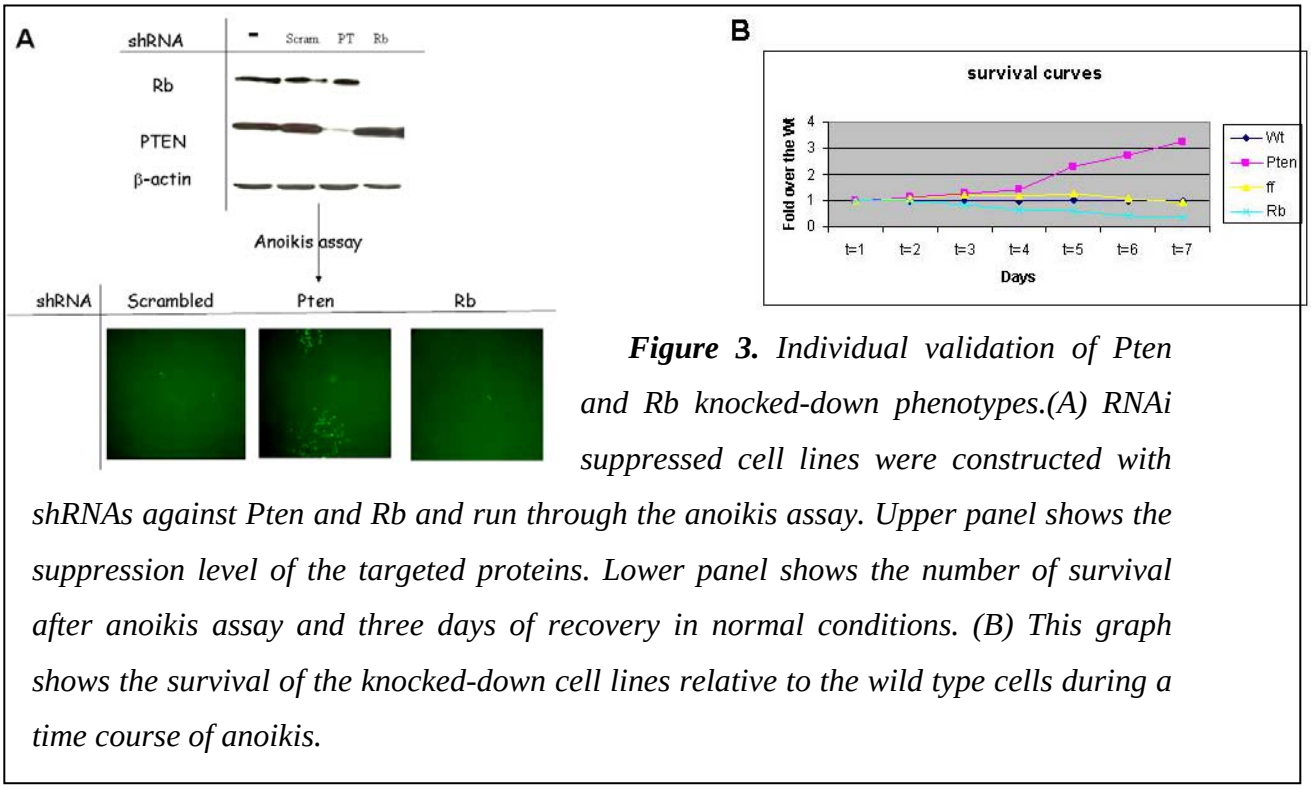

Figure 3. *Individual validation of Pten and Rb knocked-down phenotypes.(A) RNAi suppressed cell lines were constructed with shRNAs against Pten and Rb and run through the anoikis assay. Upper panel shows the suppression level of the targeted proteins. Lower panel shows the number of survival after anoikis assay and three days of recovery in normal conditions. (B) This graph shows the survival of the knocked-down cell lines relative to the wild type cells during a time course of anoikis.*

cells to apoptosis when exposed to stress stimuli[28]. Together these results validate our shRNA pool screen strategy as an adequate platform to analyze RNAi screens. They also validate our anoikis assay as an approach to identify tumor suppressors genes in breast cancer.

Key Research Accomplishments:

- Assisting in the construction and validation of second-generation shRNA (shRNAmir) expression libraries that have been designed based on an increased knowledge of RNAi biochemistry. We have generated large-scale arrayed, sequence-verified libraries comprising more than 140,000 shRNAmir expression plasmids, covering a substantial fraction of all predicted genes in the human and mouse genomes.
- A RNAi screen in MCF10A breast cancer cells identifying genes that are involved in apoptosis and growth arrest.

Reportable Outcomes:

Manuscripts:

Jose M. Silva, Mamie Z. Li, Ken Chang, Wei Ge, Michael C. Golding, Ricky Rickles, **Despina Siolas**, Guang Hu, Patrick J. Paddison, Michael R. Schlabach, Nihar Sheth, Jeff Bradshaw, Julia Burchard, Amit Kulkarni, Guy Cavet, Ravi Sachidanandam, W. Richard McCombie, Michele A. Cleary, Stephen J. Elledge, and Gregory J. Hannon. Second-Generation shRNA Libraries Covering the Mouse and Human Genomes. *Nature Genetics* 2005 Nov;37(11):1281-8.

Siolas, D., Lerner C., Burchard J., Ge W., Linsley PS., Paddison PJ., Hannon GJ., Cleary MA. (2005) Synthetic shRNAs as potent RNAi triggers. *Nature Biotechnology*. 23(2):227-31

Conclusions:

Using our shRNA pool strategy, I worked with Jose Silva, a postdoc in our lab to perform genome-wide RNAi in vitro screens and develop our microarray platform. One RNAi screen performed examined the acquisition of resistance to apoptosis and uncontrolled proliferation in normal human mammary epithelial cells (MCF-10A) upon gene suppression. We used an assay to detect resistance to anoikis (cell death trigged by loss of attachment to the extracellular matrix, ECM). Anoikis is a natural defense mechanism of monostratified epithelia against abnormal proliferation that is generally lost in transformed cells. Thus, normal cells die by anoikis when they become deattached from the ECM while tumor cells survive longer. Our screen of 1,500 shRNAs resulted in the identification of the well known tumor suppressor Pten as an antenuator of the anoikis effect.

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